Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Neutralizing Antibody against Selected Bovine Viruses
(Constant Serum-Varying Virus Method)

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Supplemental Assay Method for the Titration of Neutralizing Antibody against Selected Bovine Viruses (Constant Serum-Varying Virus Method)

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* serum neutralization (SN) assay test method to determine the neutralization index (NI) against bovine rhinotracheitis virus (IBR), bovine virus diarrhea (BVD) type I and type II, parainfluenza (PI3), or bovine respiratory syncytial virus (BRSV).

1.2 Key Words

Bovine rhinotracheitis virus; IBR; bovine virus diarrhea; BVD; parainfluenza; PI3; bovine respiratory syncytial virus; BRSV; serum neutralization; SN; antibody titer; *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] 36° ± 2°C, 5% ± 1% CO_2, high humidity

2.1.2 Vortex mixer[2]

2.1.3 Microscope,[3] inverted light

2.1.4 Microscope,[4] fluorescent

2.1.5 Micropipettor, 200 µl, 1000 µl single channel,[5] 5-50 µl x 12 channel,[6] and tips[7]

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[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
[2] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent
[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747 or equivalent
[4] Model BH2, Olympus America, Inc. or equivalent
[5] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[6] Finnpipettes®, Cat. No. NX204662D, A. Daigger Company, Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent
[7] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Center, NY 11571 or equivalent

2.1.6 Water bath[8]

2.1.7 Centrifuge[9] and rotor[10]

2.2 Reagents/supplies

2.2.1 Indictor Virus[11]

1. IBR
2. BVD type I
3. BVD type II
4. PI3
5. BRSV

2.2.2 Cell Cultures:

1. Madin-Darby bovine kidney (MDBK) cells[12] used for IBR and PI3 SN testing.

2. Embryonic bovine kidney (EBK) cells[13] used for BVD type I and type II testing.

3. Embryonic bovine lung (EBL) cells[13] used for BRSV testing.

2.2.3 Minimum essential medium (MEM)

2.2.3.1 9.61 g MEM with Earle's salts without bicarbonate[14]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[15]

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[8] Cat. No. 15-461-10, Fisher Scientific Co., 2000 Park Ln., Pittsburgh, PA 15275 or equivalent
[9] Model J6-B, Beckman Coulter, P.O. Box 3100, Fullerton, CA 92834-3100 or equivalent
[10] Type JS-4.0, Beckman Coulter or equivalent
[11] Reference quantities are available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[12] Cat. No. ATCC CCL-22, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776
[13] Available upon request from the CVB-L or equivalent
[14] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[15] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

2.2.3.3 Dissolve **Sections 2.2.3.1 and 2.2.3.2** with 900 ml deionized water (DW).

2.2.3.4 Add 5 g lactalbumin hydrolysate or edamine[16] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.3.3** with constant stirring.

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[17]

2.2.3.6 Sterilize through a 0.22-µm filter.[18]

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine[19]
2. 25 units/ml penicillin[20]
3. 50 µg/ml gentamicin sulfate[21]
4. 100 µg/ml streptomycin[22]
5. 2.5 µg/ml amphotericin B[23]

2.2.3.8 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.4.3 Store at 4° ± 2°C.

2.2.5 Maintenance Media

2.2.5.1 980 ml of MEM

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[16] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
[17] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[18] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[19] L-glutamine-200 mm (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[20] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
[21] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[22] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[23] Cat. No. A-4888, Sigma Chemical Co. or equivalent

2.2.5.2 Aseptically add 20 ml gamma-irradiated FBS.

2.2.5.3 Store at 4° ± 2°C.

2.2.6 Monoclonal antibodies (MAb)[13]

1. Anti-BVD type I MAb
2. Anti-BVD type II MAb

2.2.7 Anti-mouse fluorescein isothiocyanate labeled conjugate (Anti-Mouse Conjugate)[13]

2.2.8 80% Acetone

2.2.8.1 80 ml acetone[24]

2.2.8.2 20 ml DW

2.2.8.3 Store at room temperature (RT) (23^0 ± 2^0C).

2.2.9 0.01 M Phosphate buffered saline (PBS)

2.2.9.1 1.33 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[25]

2.2.9.2 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)[26]

2.2.9.3 8.5 g sodium chloride (NaCl)[27]

2.2.9.4 Q.S. to 1000 ml with DW.

2.2.9.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)[28] or 2N HCl.

[24] Cat. No. A 6015, Sigma Chemical Co. or equivalent
[25] Cat. No. S 0876, Sigma Chemical Co. or equivalent
[26] Cat. No. S 9638, Sigma Chemical Co. or equivalent
[27] Cat. No. S 9625, Sigma Chemical Co. or equivalent
[28] Cat. No. 925-30, Sigma Chemical Co. or equivalent

2.2.9.6 Sterilize by autoclaving at 15 psi, $121^0 \pm 2^0$C for 35 ± 5 min.

2.2.9.7 Store at $4^0 \pm 2^0$C.

2.2.10 FBS negative for IBR, BVD, PI3, and BRSV antibodies

2.2.11 Cell culture plate,[29] 96 well

2.2.12 Polystyrene tube,[30] 17 x 100 mm

2.2.13 Serological pipette,[31] 10 ml

2.2.14 Plastic wash bottle,[32] 500 ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in antibody titration assays, cell culture maintenance, and in the principles of aseptic techniques.

3.2 Preparation of equipment/instrumentation

3.2.1 On day of test initiation, set a water bath at 36° ± 2°C.

3.2.2 On day of test initiation, set a water bath at 56° ± 2°C.

[29] Costar® 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[30] Falcon® 2057, Becton Dickinson Labware or equivalent
[31] Falcon® 7530, Becton Dickinson Labware or equivalent
[32] Cat. No. 2402, Nalge Nunc Int., 75 Panorama Creek Dr., Rochester, NY 14602 or equivalent

3.3 Preparation of reagents/control procedures

3.3.1. Two days prior to test initiation, seed 96-well cell culture plates with MDBK cells, in Growth Medium, at a cell count that will produce a confluent monolayer after 2 days of incubation at $36^0 \pm 2^0$C in a CO_2 incubator. These become the MDBK Test Plates.

3.3.2 One day prior to test initiation, seed 96-well cell culture plates with EBK cells, in Growth Medium, at a cell count that will produce a confluent monolayer after a day of incubation at $36^0 \pm 2^0$C in a CO_2 incubator. These become the EBK Test Plates.

3.3.3 On the day of the test initiation, seed 96-well cell culture plates with EBL cells, in Growth Medium. These become the EBL Test Plates.

3.3.4 On day of test initiation, rapidly thaw a vial of an appropriate Indicator Virus in a $36^0 \pm 2^0$C water bath. The number of dilutions depends upon the predetermined titer of the Indicator Virus. Prepare serial tenfold dilutions as follows:

3.3.4.1 Pipette 9.0 ml of MEM with a serological pipette into 8, 17 x 100-mm polystyrene tubes labeled 10^{-1} to 10^{-8}.

3.3.4.2 Transfer 1.0 ml of an Indicator Virus to the 10^{-1} tube; mix by vortexing. Discard the pipette.

3.3.4.3 Transfer 1.0 ml from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing. Discard the pipette.

3.3.4.4 Repeat **Section 3.3.4.3** for each of the subsequent dilutions, transferring 1.0 ml from the previous dilution to the next dilution.

3.3.5 On the day of EBK Test Plate examination, dilute Anti-BVD MAb and Anti-Mouse Conjugate in PBS according to the supplied CVB-L Reference and Reagent data sheet.

3.4 Preparation of the Test Serum Samples

On day of test initiation, heat inactivate all Test Sera in a 56^0 ± 2^{0}C water bath for 30 ± 5 min.

4. Performance of the test

4.1 Add 150 µl/well of an undiluted Test Serum into a column of a 96-well cell culture plate, which becomes the Dilution Plate (**Appendix I**).

4.2. Add 150 µl/well of FBS into a column of a Dilution Plate.

4.3 Add 150 µl/well of the last 4 dilutions of the Indicator Virus to a row of the Dilution Plate.

4.4 Mix by tapping the edge of the Dilution Plate with fingers. Incubate for 60 ± 10 min at RT to allow for neutralization of the Indicator Virus.

4.5 At the end of the incubation period, decant Growth Medium from the MDBK Test Plates. **Note: The Growth Media is not removed from the EBK and EBL Test Plates.**

4.6 Inoculate 50 µl/well of each Virus-serum mixture into 5 wells/dilution of the appropriate Test Plate **(Appendix II**). **Note: MDBK Test Plates are used to determine the SN titer against IBR and PI3. EBK Test Plates are used for BVD. EBL Test Plates are used for BRSV.**

4.7 Maintain 5 or more wells on each Test Plate as uninoculated cell controls.

4.8 Inoculate 25 µl/well of the last 4 dilutions of the appropriate Indicator Virus to 5 wells/dilution as an endpoint titration.

4.9 Incubate MDBK Test Plates for 60 ± 10 min at $36^0 \pm 2^0$C in a CO_2 incubator.

4.10 Add 200 µl/well of Maintenance Medium to all wells (**do not remove virus-serum inoculum**) of the MDBK Test Plate.

4.11 Incubate the MDBK and EBK Test Plates for 96 ± 12 hr postinoculation (HPI) at $36^0 \pm 2^0$C in a CO_2 incubator.

4.12 Incubate the EBL Test Plate for 144 ± 12 HPI at $36^0 \pm 2^0$C in a CO_2 incubator.

4.13 At the end of the incubation period, examine the wells with an inverted light microscope. Record the number of wells/dilution showing any characteristic CPE for IBR, PI3, or BRSV for each Test Sera and for the FBS.

4.14 An indirect fluorescent antibody technique (IFA) is conducted to determine the SN titer against BVD as follows:

4.14.1 Decant media from the EBK Test Plates.

4.14.2 Fill wells with 80% Acetone.

4.14.3 Incubate at RT for 15 ± 5 min.

4.14.4 Decant the 80% Acetone from the EBK Test Plate; air dry at RT.

4.14.5 Pipette 35 μl/well of a diluted Anti-BVD MAb into a EBK Test Plate; incubate for 45 ± 15 min at RT.

4.14.6 Rinse by filling the wells completely with PBS; decant the liquid.

4.14.7 Repeat **Section 4.14.6** for a total of 2 washes.

4.14.8 Pipette 35 μl/well of the diluted Anti-mouse Conjugate into the EBK Test Plates; incubate for 45 ± 15 min at RT.

4.14.9 Repeat **Section 4.14.6** for a total of 2 washes.

4.14.10 Dip the plate in DW, decant; allow to air dry or dry at $36^0 \pm 2^0$C.

4.14.11 Examine wells with a fluorescent microscope.

4.14.12 A well is considered positive if typical cytoplasmic, apple-green fluorescence is observed.

4.14.13 Record the number of wells/dilution showing any characteristic fluorescence for each Test Sera and the FBS.

4.15 Calculate the $TCID_{50}$ of the Test Sera and the FBS using the Spearman-Kärber method as commonly modified.

4.16 The NI is determined by subtracting the log of the titer obtained with the Test serum from the titer obtained with the FBS.

Example: Log $TCID_{50}$ titer with FBS 6.0
Log $TCID_{50}$ titer with Test Serum - 2.7
NI = 3.3

5. Interpretation of the test results

5.1 For a valid test:

5.1.1 No visible contamination or serum toxicity should be observed in ≥ 1 well/dilution of **all** dilutions of a Test Sera or the FBS.

5.1.2 The titer of the FBS should be negative for neutralizing antibody against IBR, BVD, PI3, or BRSV.

5.1.3 The endpoint titration of the Indicator Virus should fall within 2 standard deviations of its mean titer as determined by a minimum of 10 previous titrations.

6. Report of test results

Record the NI for the Test Serum on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Parts 113.215 and 113.216, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Finney DJ. *Statistical Method in Biological Assay.* 3rd ed. 1978. Charles Griffin and Co., London.

7.3 Rose NR, H Friedman, JL Fahey, eds. *Manual of Clinical Laboratory Immunology*. Chapter 11: Neutralization Assays. 1986. ASM, Washington, D.C.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following is a listing of the significant changes made from the previous protocol:

8.1 Testing format has been changed from roller tubes and Leighton tubes to a 96-well plate format.

8.2 The provision for conducting a hemabsorption test for detecting antibody against PI3 has been replaced by detecting antibody by CPE.

8.3 The fluorescent antibody test for detecting antibody against BVD has been replaced with an IFA to allow the differentiation between type I and II BVD antibodies.

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9. Appendix

9.1 Appendix I: Dilution Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A IV 10^{-3}→	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10	TS11	FBS
B IV 10^{-4}→	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C IV 10^{-5}→	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D IV 10^{-6}→	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E IV 10^{-3}→	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20	TS21	TS22	TS23
F IV 10^{-4}→	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G IV 10^{-5}→	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H IV 10^{-6}→	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

IV = Indicator Virus dilution, TS = Test Serum, NC = FBS

9.2 Appendix II: Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 10^{-3}	TS1	TS1	TS1	TS1	TS1	CC	TS2	TS2	TS2	TS2	TS2	CC
B 10^{-4}												
C 10^{-5}												
D 10^{-6}												
E 10^{-3}	TS3	TS3	TS3	TS3	TS3	CC	TS4	TS4	TS4	TS4	TS4	CC
F 10^{-4}												
G 10^{-5}												
H 10^{-6}												

TS= Test Serum, CC= Cell Control